Ultraviolet Radiation Increases Tropoelastin mRNA Expression in the Epidermis of Human Skin *In Vivo*

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Photoaged skin contains elastotic materials in the upper reticular dermis. This phenomenon is commonly known as solar elastosis. Little is known about the mechanisms leading to the accumulation of elastotic materials in photoaged skin, however. In this study, it was demonstrated that ultraviolet irradiation induced tropoelastin mRNA expression in the keratinocytes of human skin *in vivo* and also in cultured human keratinocytes by *in situ* hybridization and reverse transcriptase polymerase chain reaction. It was also shown by northern blot analysis ($n = 5$) that there were increased tropoelastin mRNA levels in the forearm (sun-exposed) skin of elderly persons, compared with upper-inner arm (sun-protected) skin of the same individuals. As demonstrated by *in situ* hybridization compared to sun-protected skin (upper-inner arm) ($n = 5$), tropoelastin mRNA expression in photoaged skin was higher in keratinocytes as well as in fibroblasts. Therefore, our results suggest that keratinocytes are another source of tropoelastin production after acute and chronic ultraviolet irradiation in human skin *in vivo*. *Key words: aging/photoaging/solar elastosis. J Invest Dermatol 116:915–919, 2001*

Skin aging can be divided into two areas, intrinsic (chronologic) aging and photoaging (Gilchrest, 1989). The histologic findings from intrinsic aging show a general decrease in the extracellular matrix with reduced elastin and a disintegration of elastic fibers (Braverman and Fonferko, 1982). In contrast, the histologic findings of photoaged skin show the most prominent features, referred to as solar elastosis, which is characterized by the accumulation of dystrophic elastotic material in the reticular dermis (Mera *et al*, 1987; Montagna *et al*, 1989; Taylor *et al*, 1990; Warren *et al*, 1991). Little is known about the mechanisms leading to the accumulation of elastotic material in photoaged skin, although this material stains strongly with elastic tissue stains (Chen *et al*, 1986; Werth *et al*, 1996). This accumulation of elastotic material may be associated with increased elastin production in photodamaged skin. Ultraviolet B (UVB) irradiation has been demonstrated to upregulate tropoelastin gene expression both *in vivo* and *in vitro* (Uitto *et al*, 1997). Moreover, increased fibrillin expression and deposition have been reported within the reticular dermis of photoaged skin (Bernstein *et al*, 1994).

In this study, we demonstrated that acute and chronic UV irradiation induced tropoelastin mRNA expression in the epidermal keratinocytes of human skin *in vivo*. These changes may contribute to increased elastin production in photodamaged skin and accumulation of elastotic materials.

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MATERIALS AND METHODS

UV irradiation and skin samples Korean adults, volunteers without current or prior skin disease, were studied in this report. A Waldmann UV-800 (Waldmann, Villingen-Schwenningen, Germany) phototherapy device, including F75/85W/UV21 fluorescent sunlamps, served as the UV source, having an emission spectrum between 275 and 380 nm (peak at 310–315 nm). The range of the emission spectrum of the sunlamps is shown in **Fig 1**. Irradiation at the skin surface was measured with a Waldmann UV meter (Model 585100; Waldmann). The total irradiation 30 cm from the light source was 1.0 mW per cm^2. The distribution of power output was 0.5% UVC (below 280 nm), 56.7% UVB (280–320 nm), and 42.8% UVA (320–400 nm). The skin of the buttocks was irradiated with unfiltered UV and the dose that caused minimal erythema (MED) was determined 24 h after irradiation. Usually the MED measured with unfiltered UV was around 70–90 mJ per cm^2 for the brown skin of Koreans. The phototypes of Koreans include types III, IV, and V. Irradiated and nonirradiated buttock skin samples were obtained from each subject by punch biopsy. This study was approved by the Institutional Review Board at the Seoul National University Hospital, and all subjects gave written informed consent.

Keratinocyte culture in monolayers and on collagen gel Human epidermal keratinocytes were cultured in monolayers and on collagen gel as described previously (Chung *et al*, 1997a). The cultured keratinocytes were exposed to 25 mJ per cm^2 of UVR, and the cell viability at 24 h post-UV was not changed at all.

Laser-assisted microdissection Laser-assisted microdissection was performed as described by Fink *et al* (1998). The UV laser microbeam (P.A.L.M., Wolfratshausen, Germany) used for microdissection consisted of a high-beam precision nitrogen laser (wavelength 337 nm), which was coupled to an inverted microscope (Axiovert 135; Zeiss, Jena, Germany) via the epifluorescence illumination path. After microdissection of each specimen, the mineral-oil-coated cap containing the captured epidermis was placed in a microtube. RNA was extracted using a Trizol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's recommendations.

***In situ* hybridization** Digoxigenin-containing sense and antisense riboprobes to detect human tropoelastin mRNA were synthesized using T3 and T7 RNA polymerases. The 0.8 kb digoxigenin-labeled RNA probe was hydrolyzed in a solution of 30 mM sodium carbonate and 20 mM sodium bicarbonate at 60°C. *In situ* hybridization was performed on 8 μm sections as described in detail elsewhere (Fisher *et al*, 1997).

Reverse transcriptase polymerase chain reaction (RT-PCR) The epidermis was completely separated from the dermis in the 20 mM ribonucleoside vanadyl complex at 65°C for 1.5 min. The total RNA was isolated from the epidermis using a Trizol reagent. The total RNA extracted from the epidermis was reverse transcribed using a first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Germany). The resulting specific cDNA fragments were amplified with 2.5 U of Taq polymerase (Roche Diagnostics) in the presence of 20 pmol downstream primer (5′-ACCTGGGACAACTGGAATCC-3′) and upstream primer (5′-AAAGCAGCAGCAAAGTTCGG-3′) (Djavan *et al*, 1998).

To evaluate the concentration of RNA in each sample, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in the presence of 20 pmol sense primer (5′-ATTGTTGCCATCAATGACCC-3′) and antisense primer (5′-AGTAGAGGCAGGGATGATGT-3′) with an optimized number of 27 cycles. We observed that the cycle numbers for tropoelastin (34 cycles) and GAPDH (27 cycles) were in the range of linear amplification (data not shown). For each sample studied, several negative controls were performed. Reaction products were subjected to electrophoresis on 1.2% agarose gel and visualized with ethidium bromide; the signal strength was quantified by using a

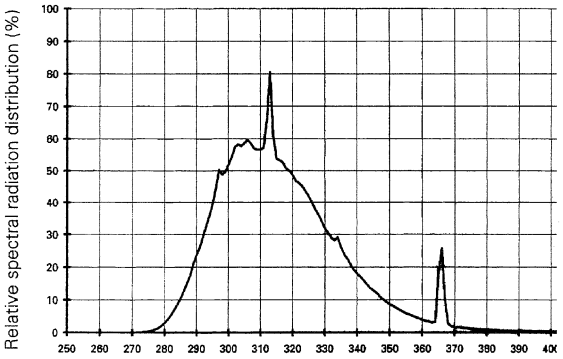

Figure 1. The emission spectra of unfiltered fluorescent sunlamps.

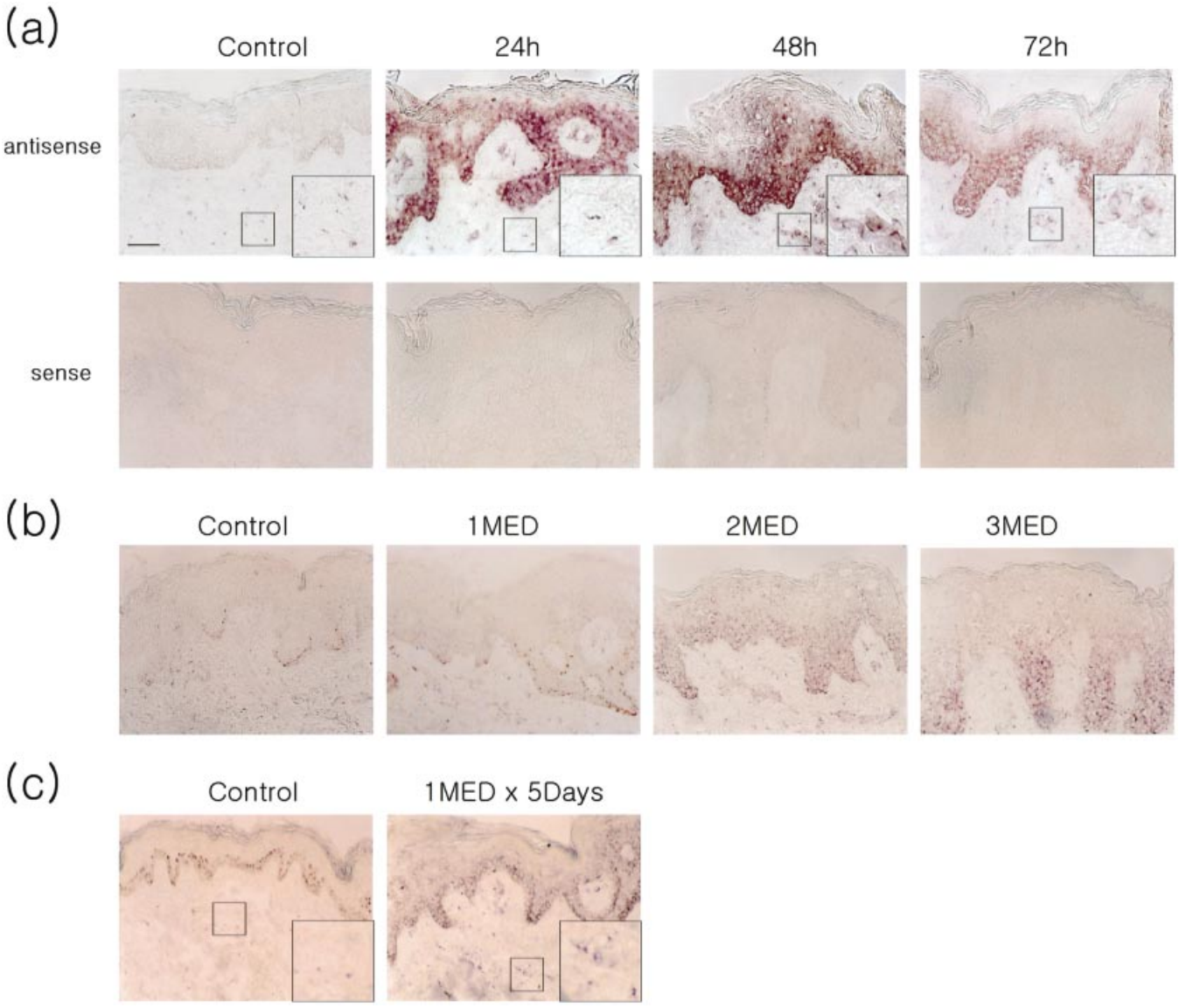

Figure 2. Acute UV irradiation induced tropoelastin mRNA expression in human keratinocytes *in vivo*. The buttock skin was irradiated with (*a*) 2 MED of UV, (*b*) 1, 2, and 3 MED of UV, and (*c*) 1 MED of UV every day for 5 d. Specimens of irradiated and nonirradiated skin were obtained at indicated time points after irradiation. Tropoelastin mRNA was detected by *in situ* hybridization. Areas outlined in boxes are shown in 2.5-fold enlargements. *Scale bar*: 25 μm.

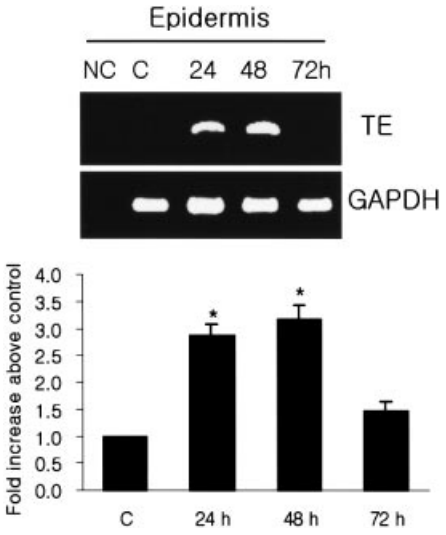

Figure 3. The expression of tropoelastin mRNA was increased in UV-irradiated epidermis of human skin *in vivo*. The buttock skin was irradiated with 2 MED of UV. Specimens of irradiated and nonirradiated skins were obtained from each subject at 24, 48, and 72 h after irradiation. The epidermis was completely separated from the dermis in 20 mM ribonucleoside vanadyl complex at 65°C for 1.5 min. Using the total RNA extracted from this epidermis, the level of tropoelastin mRNA was determined by RT-PCR. These figures are representative of the findings from five subjects. Values are the mean ± SEM of five subjects. NC, negative control; TE, tropoelastin; C, control.

densitometric program (TINA; Raytest Isotopenmeβgerate, Germany). After normalizing by GAPDH intensity, percentage increases of tropoelastin were determined. Each experiment was repeated at least three times.

Northern blot analysis Northern blot analysis was performed as described previously (Chung *et al*, 1997b). The cDNA probes were prepared by labeling the fragments of human tropoelastin (0.8 kb) and 36B4 (0.7 kb; 36B4 encodes a ribosomal protein and was used as an internal control) with [α-^{32}P]dCTP by using a Prime-It II kit (Stratagene, La Jolla, CA). The blots were exposed to a Fuji Imaging plate (Bas-2500, Fujifilm, Japan) and quantified by using a densitometric program. After normalizing for loading of the lanes as determined by 36B4 intensity, percentage increases of mRNA transcripts were determined.

Immunohistochemical staining The 8 mm punch biopsy specimens from volunteers were placed immediately into a cryomatrix (Shandon, Pittsburgh, PA) and carried to the deep freezer (–70°C). Immunohistochemical staining was performed as described previously (Chung *et al*, 1998). The polyclonal antihuman tropoelastin antibody (Elastin Products, Owensville, MO) was used for primary antibody.

Statistical analysis Statistical analyses were performed by a Mann–Whitney *U* test. A p-value less than 0.05 was considered statistically significant. All analyses were performed with Statistical Analysis Software (SAS, Cary, NC).

RESULTS AND DISCUSSION

Acute UV irradiation induced tropoelastin mRNA expression in human keratinocytes *in vivo* and *in vitro* The buttock skin was irradiated with 2 MED of UV and specimens of both irradiated and nonirradiated skins were obtained from each subject 24, 48, and 72 h after irradiation (n = 5). Tropoelastin mRNA was not detectable in the keratinocytes of nonirradiated skin after *in situ* hybridization. The expression of tropoelastin mRNA was induced 24 h after exposure to UV irradiation; it reached a maximum in all keratinocytes throughout the epidermis and was maintained through 48 h post-UV. At longer times (72 h post-UV), the expression of tropoelastin mRNA gradually

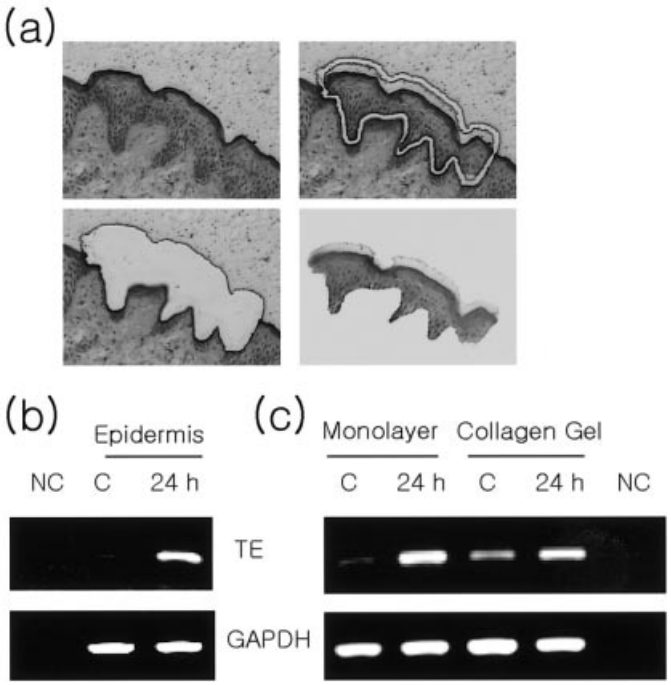

Figure 4. UV-induced expression of tropoelastin mRNA in the epidermis of human skin *in vivo* and in the cultured keratinocytes. The buttock skin was irradiated with 2 MED of UV. Specimens of irradiated and nonirradiated skin were obtained 24 h after irradiation. (*a*) Pure epidermal tissue was microdissected by laser-assisted microdissection. (*b*) Using the total RNA extracted from captured epidermal tissue, the level of tropoelastin mRNA was determined by RT-PCR. These figures are representative of the findings from three subjects. (*c*) Human epidermal keratinocytes were cultured in monolayers and on collagen gel, irradiated with 25 mJ per cm^2 of UV, and harvested 24 h after irradiation. NC, negative control; TE, tropoelastin; C, control.

decreased (**Fig 2*a***). There were many dyskeratotic cells in the upper epidermal layer 24 h post-UV, and these cells did not express tropoelastin mRNA (**Fig 2*a***). UV irradiation tended to decrease tropoelastin mRNA expression in fibroblasts 24 h post-UV (**Fig 2*a*,*insert***), however, after which there was a recovery of tropoelastin mRNA to normal levels or higher than normal at 48 and 72 h post-UVB. Hybridization of the irradiated skin with a sense probe, a control for nonspecific hybridization, yielded no detectable signal (**Fig 2*a***).

To test the effect of UV dosage, buttock skin was irradiated with 1, 2, and 3 MED. Specimens of irradiated and nonirradiated skin were obtained from each subject 48 h after irradiation (n = 3). Epidermal tropoelastin mRNA expression was induced in a dose-dependent manner (**Fig 2*b***). To determine the effect of multiple exposure, each subject was exposed to 1 MED of UV every day for 5 d. Skin specimens were obtained from each irradiated site, as well as from nonirradiated sites, 24 h after the last exposure for analysis (n = 3). Tropoelastin mRNA expression in the epidermis and in the dermal fibroblasts was found to be increased (**Fig 2*c***).

The time-dependent changes of tropoelastin mRNA expression after UV irradiation were investigated by the RT-PCR method, using the total RNA extracted from the epidermal tissues, which were separated completely from the dermis by heating (n = 5). There was very low tropoelastin mRNA expression in the control epidermis (**Fig 3**). 2 MED of UV increased the tropoelastin mRNA level in the epidermis 24 and 48 h post-UV, however. The level then decreased to the near normal level at 72 h post-UV. The PCR product was isolated, sequenced, and found to be identical to the tropoelastin cDNA fragment (data not shown). After separating the epidermis from the dermis, complete separation of epidermis was confirmed by hematoxylin and eosin stain (data not shown).

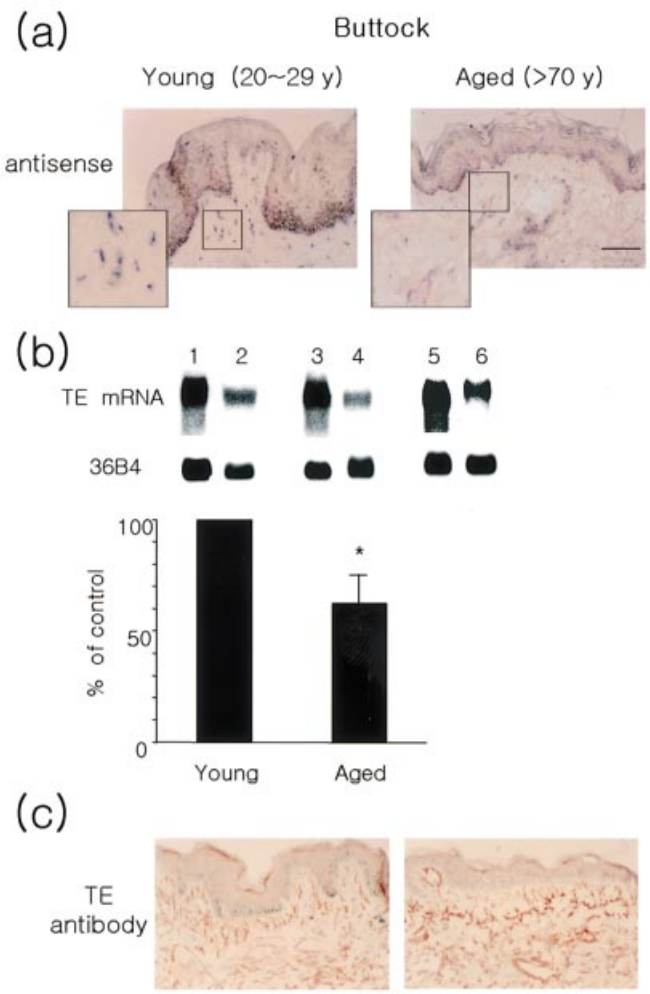

Figure 5. Tropoelastin mRNA in aged skin was decreased compared with young skin *in vivo*. Specimens of young (20–29 y) and aged (>70 y) buttock skin were obtained. Tropoelastin mRNA was measured by (*a*) *in situ* hybridization and (*b*) northern blot analyses. *Lanes 1*, *3*, *5*, young buttock skin; *lanes 2*, *4*, *6*, aged buttock skin. Values are the mean ± SEM of five subjects. *p < 0.05 *vs* young buttock skin. Elastic fibers were stained by (*c*) immunohistochemical stain. These figures are representative of the findings from five subjects. TE, tropoelastin; mean ± SEM. *Scale bar*: 25 μm.

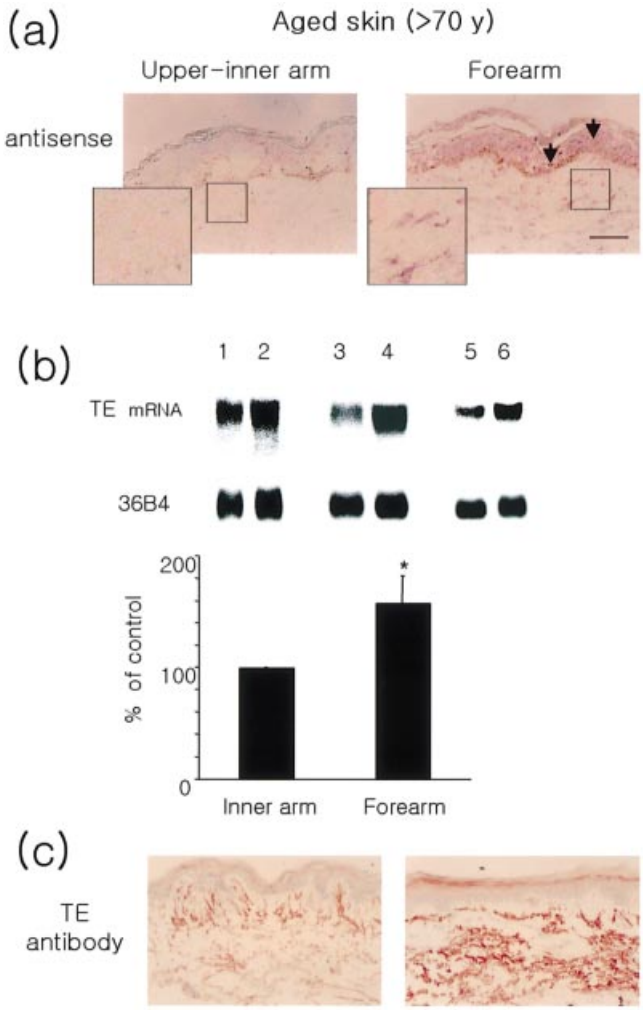

Figure 6. The expression of tropoelastin mRNA was increased in photodamaged skin compared with sun-protected skin. Specimens of forearm and upper-inner arm skin from elderly persons (>70 y) were obtained. Tropoelastin mRNA (*arrow*) was detected by (*a*) *in situ* hybridization and (*b*) northern blot analyses. *Lanes 1*, *3*, *5*, upper-inner arm skin; *lanes 2*, *4*, *6*, forearm skin. Values are the mean ± SEM of five subjects. *p < 0.05 *vs* old sun-protected skin. Elastic fibers were stained by (*c*) immunohistochemical stain. These figures are representative of the findings from five subjects. TE, tropoelastin.

To confirm the expression of tropoelastin mRNA in the keratinocytes *in vivo*, the epidermis was carefully microdissected to avoid contamination with dermal components using laser-assisted microdissection ($n = 3$) (**Fig 4*a***). RT-PCR was then performed using the total RNA extracted from the captured epidermal tissues. Again there was very low tropoelastin mRNA expression in the control epidermis. 2 MED of UV increased epidermal tropoelastin mRNA expression 24 h post-UV (**Fig 4*b***).

UV (25 mJ per cm^2) increased the tropoelastin mRNA expression in cultured human epidermal keratinocytes ($n = 3$). Tropoelastin mRNA expression could be detected in nonirradiated keratinocytes, and was increased at 24 h post-UV (**Fig 4*c***). To investigate whether the culture methodology had an effect on tropoelastin mRNA expression, keratinocytes were cultured three-dimensionally on a collagen matrix without fibroblasts. The control keratinocytes, cultured on the collagen gel, showed low tropoelastin mRNA expression ($n = 3$) (**Fig 4*c***). UV irradiation could also increase tropoelastin mRNA expression in keratinocytes cultured on collagen gel.

Under normal circumstances, epithelial cells such as keratinocytes are not considered as elastin-producing cells. Our results demonstrate that human epidermal keratinocytes are able to produce tropoelastin by certain environmental stimuli such as UV. Starcher *et al* (1999) also reported that in hairless mice UV irradiation increased both the number and size (length and diameter) of elastic fibers in the dermis, and that modified epithelial cells surrounding the hair follicles and sebaceous glands were the source.

Elastic fibers are insoluble structural elements of connective tissues that have a central core of amorphous, hydrophobic cross-linked elastin surrounded by fibrillin-rich microfibrils (Mecham and Heusar, 1991). Fibrillin is a product of dermal fibroblasts. Keratinocytes also express fibrillin and assemble microfibrils, however (Haynes *et al*, 1997). Recently, keratinocytes have been shown to influence the maturation and organization of the elastin network in a skin equivalent model (Duplan-Perrat *et al*, 2000). Our finding in this study suggests that keratinocytes may contribute to elastin network formation by producing tropoelastin in human skin *in vivo*.

Tropoelastin mRNA was increased in the keratinocytes and fibroblasts in photodamaged skin *in vivo* In young skin, as

measured by *in situ* hybridization ($n = 5$), tropoelastin mRNA was strongly expressed in the dermal fibroblasts but not in the keratinocytes. In aged buttock skin, however, there was a dramatic decrease of tropoelastin mRNA expression in the fibroblasts and there was no expression in the keratinocytes ($n = 5$) (**Fig 5*a***). To quantify the tropoelastin mRNA level, total RNA was extracted directly from the punch biopsy specimens of buttock skin from both young and elderly subjects. The tropoelastin mRNA levels, measured by northern blot analyses, were significantly lower in aged skin ($n = 5$), by an average of 40%, compared with young skin ($n = 5$) (**Fig 5*b***).

Specimens of both forearm and upper-inner arm skin from elderly persons (>70 y) were obtained. In this case after *in situ* hybridization minimal amounts of tropoelastin mRNA in the fibroblasts were detected in the sun-protected skin ($n = 5$) and there was no tropoelastin mRNA expression in the keratinocytes. In sun-exposed skin ($n = 5$), however, tropoelastin mRNA expression increased significantly not only in the fibroblasts but also in the keratinocytes (**Fig 6*a***), compared with the sun-protected skin of the same individuals. To quantify the tropoelastin mRNA level, the total RNA was extracted directly from punch biopsy specimens of both forearm and upper-inner arm skin from elderly subjects. The tropoelastin mRNA levels were determined by northern blot analysis. The forearm skin demonstrated increased tropoelastin mRNA expression compared with the sun-protected upper-inner arm skin from the same individual ($n = 5$), after correction for the 36B4 levels (**Fig 6*b***).

Bernstein *et al* (1994) also reported increased elastin mRNA from biopsy samples in photodamaged skin. In our study, it was shown that tropoelastin mRNA expression was higher in both the keratinocytes and fibroblasts of photodamaged skin (forearm) compared with sun-protected skin (upper-inner arm). Our results suggest that keratinocytes in photoaged skin, as in acutely irradiated skin, may be a source of tropoelastin in addition to the dermal fibroblasts in human skin *in vivo*.

It is well known that UVB can upregulate tropoelastin mRNA expression in cultured fibroblasts (Bernstein *et al*, 1994; Uitto *et al*, 1997). Other investigators have reported that UVB can down-regulate tropoelastin mRNA expression in cultured fibroblasts, however (Werth *et al*, 1997). Our results indicate that acute UV irradiation decreased tropoelastin mRNA expression initially 24 h post-UV in the dermal fibroblasts of human skin *in vivo*. Chronic UV exposure of human skin, as observed in the photoaged skin of the elderly, seems to upregulate tropoelastin mRNA expression in the dermal fibroblasts *in vivo*. The reasons for the opposite effects of acute and chronic UV exposure on tropoelastin expression remain to be investigated further. It is possible, however, that various cytokines and growth factors produced by inflammatory cells in chronically photodamaged skin may play some role in the stimulation of fibroblasts to produce more tropoelastin.

By immunohistochemical staining, oxytalan fibers were significantly fewer in aged buttock skin, but the elastic fibers in the upper and mid-dermis became thicker and more fragmented, compared with those of young skin ($n = 5$, **Fig 5*c***). In sun-exposed skin, however, there were few disrupted oxytalan fibers in the papillary dermis and large amounts of elastotic material in the reticular dermis ($n = 5$, **Fig 6*c***).

The mechanisms for the loss of microfibrillar integrity in the upper dermis in photoaged skin remain to be delineated. Chronic UV exposure can induce dermatoheliosis and cause chronic inflammatory cell infiltration. These inflammatory cells produce various proteinases such as neutrophil elastase and neutrophil collagenase, which can degrade intact microfibrils rapidly (Kielty *et al*, 1994). It is well known that matrix metalloproteinases, such as collagenase and gelatinase, can be induced by UV irradiation in human skin *in vivo*, and these are also enhanced in photoaged skin (Fisher *et al*, 1997). It is therefore possible that these enzymes may play important roles in disrupting the elastic fiber network in photodamaged skin.

Thus, it may be suggested that the abnormal production of tropoelastin in both fibroblasts and keratinocytes by UV, and its deposition and degradation by various enzymes in the upper dermis, may partially contribute to the accumulation of elastotic materials in photoaged skin.

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